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Probing sugar translocation through maltoporin at the single channel level

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Abstract Sugar permeation through maltoporin of *Escherichia coli*, a trimer protein that facilitates maltodextrin translocation across outer bacterial membranes, was investigated at the single channel level. For large sugars, such as maltohexaose, elementary events of individual sugar molecule penetration into the channel were readily observed. At small sugar concentrations an elementary event consists of maltoporin channel closure by one third of its initial conductance in sugar-free solution. Statistical analysis of such closures at higher sugar concentrations shows that all three pores of the maltoporin channel transport sugars independently. Interestingly, while channel conductance is only slightly asymmetric showing about 10% higher values at −200 mV than at +200 mV (from the side of protein addition), asymmetry in dependence of the sugar binding constant on the voltage polarity is about 20 times higher. Combining our data with observations made with bacteriophage-λ we conclude that the sugar residence time is much more sensitive to (and is decreased by) voltages that are negative from the intra-cell side of the bacterial membrane.

Key words: Sugar transport; Ion channel reconstitution; Current fluctuation; Noise analysis

1. Introduction

The outer membrane of Gram-negative bacterium *Escherichia coli* serves as a barrier against harmful components but allows the uptake of nutrients through several pore-forming proteins [1]. Most abundant are the non-specific diffusion pores that allow the uptake of small ions and small polar solutes up to 600 Da. In addition to these general diffusion channels, the outer membrane also contains proteins that form channels with high substrate specificity. The most extensively studied example of specific porins is the maltodextrin-specific channel LamB or maltoporin. Maltoporin is expressed as part of the mal-operon upon induction by maltose or maltodextrins, and serves as the receptor for bacteriophage-λ. The facilitated maltodextrin translocation across maltoporin has been shown by liposome swelling assays [1,2].

Recently the 3D structure of the trimeric maltoporin has been elucidated [3–5]. The monomer of LamB of *E. coli* consists of an 18-stranded β-barrel with short turns on the periplasmic side and large irregular loops on the outside of the cell. The third loop, L3, folds inside the β-barrel forming a constriction in the middle of the channel. Based on the sugar-soaked structure of maltoporin, a specific sugar translocation pathway consisting of an aromatic amino acid slide aligned by polar track residues has been postulated [3,5]. The hydrophobic faces of the sugar rings are in van der Waals contact with the greasy slide, while an additional interaction is due to the hydrogen bonds between the sugar hydroxyl groups and the polar track residues.

The kinetics of sugar translocation has been addressed in several studies [6–9] that employed noise analysis of large populations of maltoporin channels. It was shown that the addition of sugars reduced the average conductance of the channel population and induced excess noise that often contained a Lorentzian component. The magnitude of this reduction and the parameters of the Lorentzian component permitted evaluation of sugar binding rates based on the assumption that sugar molecules bind independently to each monomer of the channel trimer.

Here, using time-resolved measurements of maltohexaose binding to a single maltoporin channel, we show that the binding is, in fact, independent. Our approach reveals fine molecular details of the process. We quantify the voltage-dependent transport properties of the maltoporin channel. We demonstrate that the channel is asymmetric in both sugar and ion transport and is always inserted in an oriented manner. Surprisingly, we find that while asymmetry of the channel in ion conductance is rather weak, the voltage-induced asymmetry in sugar translocation is very pronounced.

2. Material and methods

Planar lipid bilayers were formed using the lipid monolayer opposition technique from 0.25% solution of diphytanoylphosphatidylcholine (Avanti Polar Lipids, Alabaster, AL, USA) in pentane (Burdick and Jackson, Muskegon, MI, USA) on a 70 μm diameter aperture in the 15 μm thick Teflon partition as previously described [10,11]. The total capacitance was 50–60 pF, the film capacitance was close to 25 pF. Small amounts of wild type maltoporin from a diluted stock solution of 1 mg/ml containing 1% (v/v) of OctylPOE from Alexis, Switzerland, were added to the *cis*-side of the chamber. The final concentration of the porin in the membrane-bathing solution (1 M KCl, 1 mM $CaCl_2$ and 10 mM Tris buffered to pH 7.4) was about 0.1 pM. Maltoporin insertion into the lipid bilayer was favored by applying a positive external transmembrane voltage of 100–200 mV to the *cis*-side, which is the side of maltoporin addition. Maltohexaose was purchased from Sigma (St. Louis, MO, USA). All experiments were done at room temperature, $T = (23.0 \pm 0.5)$°C.

Conductance measurements were performed using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA) in the

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voltage clamp mode. Data were filtered by a low-pass 8-pole Butterworth filter (Model 9002, Frequency Devices, Haverhill, MA, USA) at 15 kHz and recorded simultaneously by a VCR operated in digital mode and directly saved into the computer memory with a sampling frequency of 50 kHz. Amplitude and power spectrum analysis was done using software developed in-house.

3. Results and discussion

Understanding the physical basis of channel selectivity to metabolites necessarily requires detailed information on molecular kinetics. One of the powerful kinetic methods is noise analysis of multi-channel membranes [12–14] which was successfully applied in metabolite transport studies [6–9]. Now, with the progress in small signal detection and analysis, we are able to study the transport of polymers and metabolites at the single channel level [15].

Fig. 1 shows current recordings obtained from a single maltoporin trimer in the presence of maltohexaose of varying concentration. The events of single molecular binding are seen as current interruptions due to transient pore occlusions by a sugar molecule. At small sugar concentrations, sugar binding results in a drop in the current to 2/3 of the initial value, suggesting that only one of the monomers is blocked for K^+ and Cl^- ions. At higher sugar concentrations, double and triple blocking is evident. One maltohexaose molecule completely occludes ion permeation. Statistical analysis shows that when all three monomers are blocked by sugars simultaneously, the residual current is zero within the accuracy of our measurements (0 ± 0.4 pA). Thus, the effect is complete up to the accuracy of 2%.

The resolved single molecule events make it possible to test the binding independence hypothesis. If binding to different monomers comprising the channel is independent and monomers are identical, one would expect that the distribution of probabilities to find the channel in a particular blocked state (2/3, 1/3 or 0/3 of the initial conductance) is described by a binomial distribution. Indeed, if p is the probability to find a given monomer of the channel trimer in a blocked state, then the probability to find zero, one, two or three monomers blocked simultaneously is given by:

$$P_k = \frac{3!}{K!(3-k)!} p^k (1-p)^{3-k} \quad (1)$$

where k is the number of monomers blocked and p is defined through maltohexaose bulk concentration, [M], and equilibrium constant of binding, K, as:

$$p = \frac{K[\mathrm{M}]}{K[\mathrm{M}] + 1} \quad (2)$$

Fig. 2 compares the prediction of Eq. 1 with experimental data obtained from a single maltoporin channel in the presence of different sugar concentrations. The experimental points were obtained from amplitude distribution histograms of current records, examples of which are shown in Fig. 1. It is seen that the probability of the fully open state monotonically decreases with sugar concentration, while the probability of the completely blocked state monotonically increases. The probability of one state being closed goes through a maximum at sugar concentrations around 30 μM. The non-monotonic character of the probabilities for the simultaneous blocking of

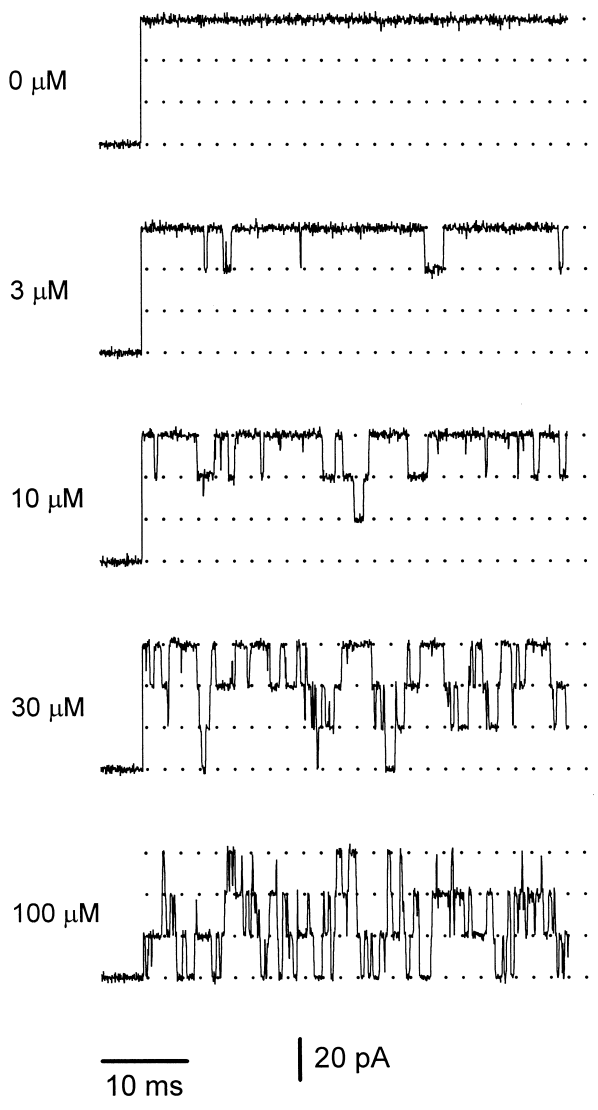

Fig. 1. Current recordings of a single maltoporin channel at different maltohexaose concentrations (given as numbers on the left) demonstrate time-resolved events of the reversible sugar binding. At small sugar concentration (e.g. 3 μM) short interruptions in the channel current by one third of its initial value in sugar-free solution are seen. As the sugar concentration is increased, current interruptions in the different monomers comprising the trimer channel overlap displaying two thirds (10 μM recording) or even complete (30 and 100 μM recordings) transient blockages of the channel current. Transmembrane voltage of +200 mV is applied from the side of protein addition. Current records are presented at 50 μs time resolution.

one or two monomers is intuitively clear. At sufficiently small sugar concentrations virtually none of the three monomers is blocked, while at sufficiently high sugar concentrations all three are blocked for most of the time.

All four theoretical curves in Fig. 2 use the same value of equilibrium constant $K = (79\ \mu\mathrm{M})^{-1}$. This value is in good correspondence with the one reported by Benz et al. [8]. The bulk concentration of maltohexaose equal to 79 μM will reduce the average current through the maltoporin trimer by a factor of two. It is seen that the binomial distribution given by Eqs. 1 and 2 describes our experimental findings perfectly. The conclusion we draw is that, within the accuracy of our

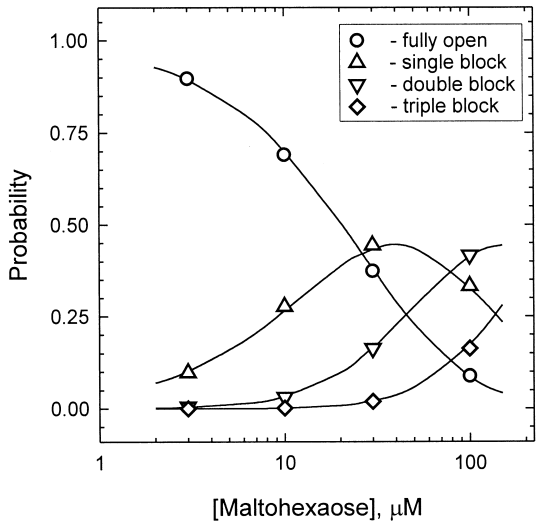

Fig. 2. The probability of finding the channel in a particular state is described by a binomial distribution (solid lines, Eqs. 1 and 2). This finding strongly suggests independence of sugar binding to (and translocation through) the different monomers of the trimeric channel. The probabilities were found as the ratios of the total time spent by the channel in a particular state to the total observation time. The size of the symbols equals or exceeds the statistical error. More than 10^5 blocking events were analyzed to obtain necessary statistics.

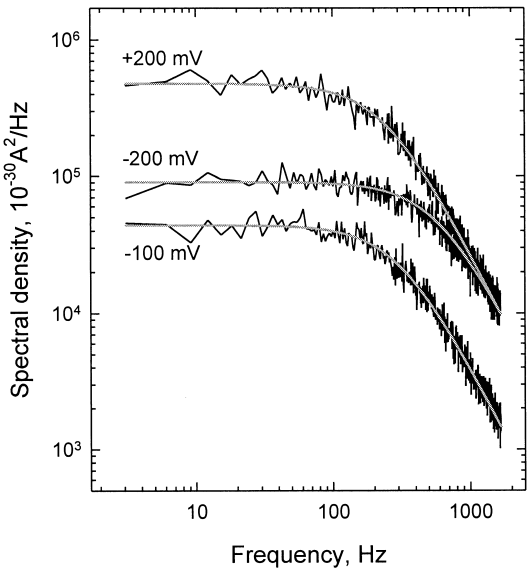

Fig. 3. Power spectral densities of fluctuations in the current through single channels show Lorentzian spectra with both the amplitude and the characteristic corner frequency depending on the applied voltage. The spectra imply that sugar binding can be described by a simple two-state Markovian process. They also indicate that the channel is asymmetric; negative voltages correspond to higher characteristic frequencies. Maltohexaose concentration is 26 μM.

experiment, sugar translocation through different monomers of the maltoporin trimer is mutually independent.

Kinetic information for sugar binding can be obtained from the examination of times spent by a monomer in a blocked state. This can be done in two ways. A straightforward approach is to analyze the time distribution histograms of records such as in Fig. 1. The other way is to determine the frequency content of the sugar-induced current fluctuations, that is, to measure current power spectrum. Then, if this spectrum has a simple Lorentzian form, the characteristic 'corner frequency', f_c, defined as the frequency at which the spectral density is two-fold decreased, gives the relaxation time of sugar binding by $\tau = 1/(2\pi f_c)$. For a simple first-order binding the bound sugar residence time, τ_r, is given by:

$$\tau_r = \tau/(1-p) \quad (3)$$

where, due to monomer independence shown above, the term in brackets can be readily found as the ratio of the average channel conductance in the presence of sugar to its conductance in sugar-free solution. From Eq. 3 it is seen that at sufficiently low sugar concentrations these two times approach each other because the blocking probability p goes to zero.

Fig. 3 shows current noise power spectra taken at three different voltages. It is seen that an increase in the applied voltage increases current noise. Corner frequencies and plateau values of the Lorentzians (smooth solid curves through the data) are: 310 Hz and 4.4×10^{-26} A^2/Hz for −100 mV; 586 Hz and 9.1×10^{-26} A^2/Hz for −200 mV and 242 Hz and 4.8×10^{-25} A^2/Hz for +200 mV. Importantly, there is a pronounced asymmetry with the sign of the applied voltage. Comparing noise spectra obtained for +200 and −200 mV one can see that the rate of sugar exchange as characterized by the corner frequency is about 2.5 times larger at the negative potential.

In most cases, the spectra were remarkably close to a single Lorentzian form over the frequency range of three decades. According to the structural data, sugar interaction with the channel includes several elementary steps happening in succession: diffusion from the bulk to the channel, binding to and orienting by the van der Waals forces of the 'greasy slide' and by hydrogen bonds between the sugar hydroxyl-groups and the polar track residues [3,5], moving along the slide and,

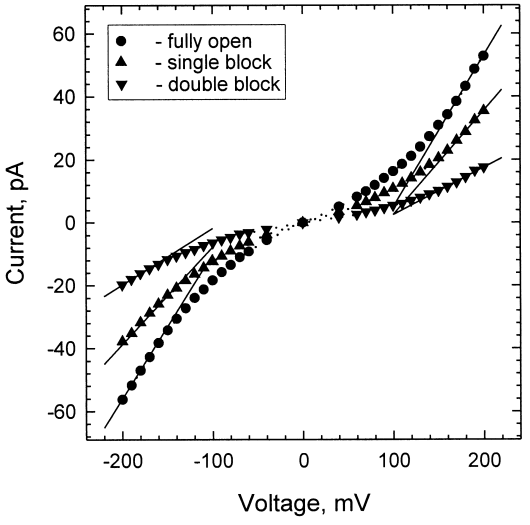

Fig. 4. Current–voltage relationships for different channel states display superlinear behavior. The differential conductance of the whole channel (and of each of the monomers) grows more than three-fold when the voltage is increased from 0 to 200 mV. It also demonstrates that the channel is slightly asymmetric in small-ion conduction; integral conductance is somewhat higher at negative potentials (see text). Maltohexaose concentration is 10 μM.

finally, release from the other (or the same) side of the channel. Surprisingly, the pure Lorentzian shape of the spectra suggests that this whole succession can be perfectly described by a two-state Markovian process. It also supports the binding independence assertion (made above from the binding equilibrium data in Fig. 2) on the dynamic level.

Random sugar binding allows us to determine maltoporin channel current–voltage characteristics for every state of the channel from its fully open trimer to a single open monomer. These results are shown in Fig. 4. Current I is superlinear in transmembrane voltage V in both positive and negative branches. For all states the slope of the current–voltage characteristic (i.e. dI/dV, solid curves in Fig. 4) increases by a factor of 3.5 ± 0.3 when the voltage is increased from 0 to +200 mV.

By studying channels at different voltages we have also found that the channel is asymmetric in its ion conduction and its insertion is always directional. The channel asymmetry can be assessed from Fig. 4. Conductance of the channel (I/V) is not only voltage-dependent, but is slightly larger at negative voltages (i.e. when the potential of the *cis*-side, which is the side of the maltoporin addition, is smaller). We have observed this asymmetry in every insertion where we checked it (150 insertions total). In the particular example in Fig. 4 at ± 200 mV this conductance asymmetry is close to 6%.

The voltage-induced asymmetry for the sugar binding is much higher. Fig. 5 presents the characteristic residence time calculated by Eq. 3 as a function of the applied voltage. About three-fold difference is seen between −200 and +200 mV of applied voltage. The sugar molecule used in this study, maltohexaose, is neutral, so the mechanism of this voltage dependence is not clear at the moment. There are many possibilities that are discussed in the current literature [16]. We speculate that the applied field induces some sign-dependent structural changes that are of minor importance for the transport of small ions, K^+ and Cl^- but, however, are significant for the sugar translocation.

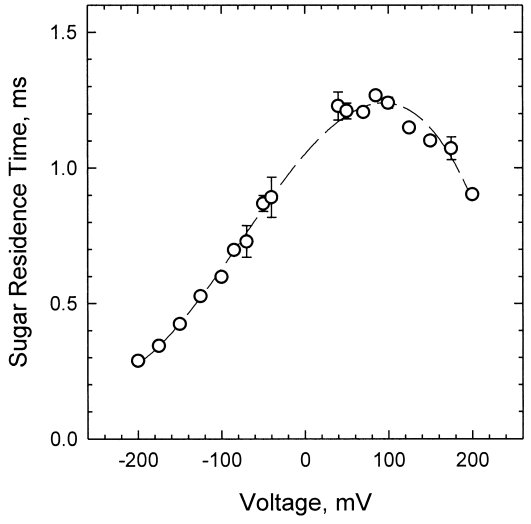

Fig. 5. Sugar binding shows a pronounced channel asymmetry. The binding residence time, τ_r, changes from 0.3 ms at −200 mV to 0.9 ms at +200 mV, going through a maximum of 1.3 ms at about +70 mV. The residence time was calculated from the binding relaxation time and the binding probability (both measured at 10 μM of maltohexaose) using Eq. 3.

Both the ionic and the sugar transport properties show that in our reconstitution protocol the channel insertion is directional. It is interesting to link this orientation to the native maltoporin orientation in the cell. To answer this question we performed experiments on bacteriophage binding. Maltoporin is known to be the receptor for bacteriophage-λ, which binds to the extracellular loops of the maltoporin and thus allows determination of the reconstituted channel orientation in the artificial bilayer.

In a bacteriophage binding assay after reconstitution of 30–50 maltoporin channels into the bilayer we carefully removed the unbound protein from the chamber by flushing with fresh protein-free membrane-bathing solution (Section 2) containing 10 mM $MgCl_2$. Bacteriophage was added to the *trans*-side of the membrane and a small reduction in conductance was detectable upon binding. In contrast to preparation in the absence of the phage, the addition of maltohexaose did not decrease the conductance. Thus, the blocking of the channel by maltohexaose was inhibited by bacteriophage. Addition of 10 mM EDTA causing the bacteriophage to unbind, led to an immediate reduction of the conductance as the sugar could now penetrate the channel constriction. Control measurements performed by adding the bacteriophage on the opposite (*cis*-) side did not influence the blocking of the channel. We conclude that, in what concerns channel orientation, the *cis*-side of the lipid bilayer in our experiments corresponds to the inner side of the bacterial membrane.

4. Conclusions

1. Single molecular events of maltohexaose binding/translocation can be observed as time-resolved transient interruptions in the ion current through the maltoporin channel.
2. One sugar molecule completely blocks one of the monomers of the trimer; when all three monomers are blocked the residual current is zero (smaller than 2% of the current for the unblocked channel).
3. Monomers bind sugars independently of each other, which results in a binomial distribution of the probabilities to find the channel in different conductance states.
4. At the conditions described, maltoporin insertion is always directional (150 times from 150 attempts in different experiments) with the extracellular loops of the channel facing *trans*-side of the membrane.
5. The channel is slightly asymmetrical in its conductance (about 10% difference at +200 mV versus −200 mV); in contrast, voltage-induced asymmetry in its sugar transport is strongly pronounced (about 200% difference in the sugar residence time at these voltages).

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